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(71) Applicant *(for all designated States except US)*: INSTITUTE FOR ANIMAL HEALTH LIMITED [GB/GB]; Compton, Newbury, Berkshire RG20 7NN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BARROW, Paul, Andrew [GB/GB]; (GB). TURNER, Arthur, Keith [GB/GB]; Institute for Animal Health Limited, Compton, Newbury, Berkshire RG20 7NN (GB).

(74) Agent: BASSETT, Richard; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).

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(54) Title: VACCINE COMPOSITIONS

(57) Abstract

A vaccine composition comprising an avirulent mutant of a cellular pathogen which colonizes the vertebrate gut, the mutant being characterised by having a functional deletion of a gene encoding a protein involved in the electron transport chain or ATP synthase. The pathogen may be *Salmonella* or *E. coli*. The vertebrate may be calves or chicks. The gene may be a *nuo* (encoding a sub-unit of NADH dehydrogenase I) or a *cyd* (encoding a cytochrome) gene. The mutants provoke an immune response and also inhibit colonization of the gut by other pathogens.

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VACCINE COMPOSITIONS

The present invention relates to vaccine compositions comprising attenuated pathogens.

Background

We discovered in the middle 1980's (Barrow *et al*, 1987) that oral inoculation of newly hatched chickens with a *Salmonella* strain resulted in massive intestinal colonization. This prevented establishment of a second strain given orally 24 h later. The effect was genus specific such that colonization with *E. coli* or other closely related genera such as *Citrobacter* did not prevent gut colonization by *Salmonella* and *vice versa*. An *in vitro* model was developed in which 24 h nutrient broth cultures of a *Salmonella* are inoculated with small numbers of a closely related strain or with the same strain with a different marker. If the mixed culture is reincubated the second strain does not grow. However, if the first strain is *E. coli* and the second strain *Salmonella*, the *Salmonella* does grow (and *vice versa*). We carried out further work to try to characterise in more detail the practical aspects of the inhibition *in vivo* (Berchieri & Barrow, 1990) and *in vitro* (Berchieri & Barrow, 1991).

We have now taken this work further and have developed vaccine compositions comprising attenuated pathogens.

Summary of the Invention

One aspect of the invention provides a vaccine composition comprising an avirulent mutant of a cellular pathogen which colonizes a vertebrate mucosal surface (preferably the gut), the mutant being characterised by

having a functional deletion of a gene encoding a protein involved in the electron transport chain or ATP synthase.

We have found that such mutants provide effective attenuated/avirulent strains for raising an immune response whilst not causing serious disease, and also (in at least some cases) provide an exclusion effect in the mucosal surface, thereby inhibiting the growth of other (non-attenuated) strains of the same pathogen or other pathogens.

Surprisingly, such mutants do not exhibit this exclusion/inhibition effect in the *in vitro* model discussed above. Hence, Zambrano and Kolter (1993) disclosed that *E. coli* mutants (*nuoA* or *nuoB*) lacking NADH dehydrogenase I had a competitive disadvantage in stationary phase, which would not have suggested their use in a vaccine. Mutants with deletions in other genes (for example *aroA* (Griffin & Barrow (1993) *Vaccine* **11**, 457-462; Barrow *et al* (1990) *Epidemiol. Infect.* **104**, 413-426) and *his pur*) are satisfactorily attenuated but do not exhibit the inhibition of colonization by other strains/pathogens.

The electron transport chain and associated F_0F_1 ATP synthase are common to all organisms which respire and it is reasonable to suppose that the invention, demonstrated below in relation to *E. coli* and *Salmonella typhimurium*, is applicable to all cellular pathogens, for example bacteria, fungi and protozoa. The pathogen may, for example, be any Eubacterial pathogen, such as any of the *Vibrio* spp., *Campylobacter* spp., *Neisseria* spp. or *Mycobacterium* spp. Preferably, however, it is *E. coli* or a Salmonella, such as *Salmonella typhimurium*, *S. enteritidis* or *S. gallinarum*. The pathogen may generally be one which is transmitted vertically (ie from mother to offspring).

A protein is "involved in" the electron transport chain if functional absence of the protein selectively damages the operation of the electron transport chain.

The genes involved in the electron transport chain include those encoding all or a subunit of or regulating the function of NADH dehydrogenase I, flavoproteins, coenzyme Q and cytochromes such as cytochromes b, c_1, c, a and a_3. Preferably, the gene encodes a pyridine-linked dehydrogenase such as an NADH dehydrogenase I or an NADPH dehydrogenase. In the operon for ATP synthase, *uncH* is a suitable gene for mutation. Many genes have already been identified as encoding a protein involved in the electron transport chain, for example all of the *E. coli nuo* genes encoding the various subunits of NADH dehydrogenase I. In addition, we disclose below the sequences of the *S. typhimurium nuoG* and *nuoH* genes. The invention may, of course, be applied to genes which have yet to be identified.

The mucosal surface which the pathogen colonizes is preferably the gut. In newly-hatched chickens, colonization of the gut by bacteria is extensive. Later, the main site is the lower end of the alimentary tract, where the flow rate of contents is slower. The crop is also colonized, albeit to a lesser extent. The organisms generally exist in the lumen and may have an association with the mucus which allows inoculation of fresh chyme as it enters the caeca (chick) or colon/caecum (calf).

Commercially, gut pathogens are particularly important in the rearing of calves, pigs, lambs and chickens, but the invention is generally applicable to any vertebrate, particularly mammals (including man) and birds (for example turkeys and ducks). The vaccines of the invention may be especially valuable in the protection of agammaglobulinaemic calves

(which have not acquired enough maternal IgG from the colostrum) against bacterial septicaemias. In a human context, the vaccines may be especially useful if the intestine is colonized by antibiotic-resistant organisms, such as *Pseudomonas* or *Staphylococcus aureus* following antibiotic therapy prior to bowel surgery.

The mutants may be made by any convenient means, for example by transposon mutagenesis using Tn *phoA* or bacteriophage P22, followed by appropriate screening, by site-directed mutagenesis or by insertion of anti-sense DNA. The mutation may cause the gene to produce no protein at all, for example by introducing a stop codon early in the coding sequence or by interfering with the promoter or some other regulatory region (including a gene which produces a factor that causes or enhances expression of the electron transport gene). Alternatively, it may cause non-functional protein to be produced.

The vaccine composition may be formulated and administered in any conventional way; administration to the gastrointestinal tract, for example by nasal spray or oral drench, is preferred to parenteral administration. The most preferred method (at least for chicks) is to spray them with an aqueous preparation of the vaccine containing 10^5-10^7 cfu/ml of the mutant organism so that each chick receives 10^3-10^5 cfu (colony forming units) by taking the drops off its fluff. The vaccines of the invention may be particularly useful if administered early (ie immediately after hatching) to chicks, for example to prevent or ameliorate infections caused by vertical transmission in hatcheries. Breeders and layers may be revaccinated by administering i.m. 0.05 ml containing about 10^5-10^7 cfu per dose of a killed vaccine at, say, twelve to sixteen weeks.

A further aspect of the invention provides the newly-isolated *Salmonella*

nuoG and *nuoH* genes or variants thereof. Such genes are useful in designing constructs for deleting the genes.

Preferred aspects of the invention will now be described by way of example and with reference to the accompanying drawings, in which:

Figure 1 shows partial sequences of the *nuoG* gene and entire coding sequence of the *nuoH* gene of *S. typhimurium* F98. The sequence starts at residue 1840 of the sequence previously reported by Archer *et al* (3). Beneath the nucleotide sequence is an alignment of the deduced amino acid sequences of the *nuoG* and *nuoH* gene products (subunits of NADH dehydrogenase I) from *S. typhimurium* and *E. coli*, showing only residues that vary between the two species (identical residues being indicated by a dot). The putative *S. typhimurium nuoG* gene product contains an additional nineteen amino acids at the C-terminus not present in the *E. coli* homologue. Putative Fe-S clusters in the *NuoG* sequence are underlined. A putative ribosome-binding site (Shine-Dalgarno sequence) is double underlined.

Figures 2 shows the strategy used to generate *S. typhimurium* defined mutants of *nuo*:Km.

Figure 3 shows the inhibitory activity of 24 h LB cultures of *S. typhimurium* mutant AB145 (A, closed circles) or F98 (B, open circles) for F98 Spc^r (open and closed diamonds) after incubation for 1-3 days.

Figure 4 shows the oxidase activity of NADH dehydrogenase from the F98 wild-type strain and the F98 *nuoG*::Tn*phoA* mutant. Membrane vesicles were prepared from F98 wild-type (closed symbols) and the *nuoG*::Tn*phoA* mutant (open symbols) and were assayed for oxidation of

NADH (upper) and dNADH (lower). Results were normalised to 1 and are therefore presented as relative absorbance.

Figure 5 (on 6 sheets) shows the sequence of the *Salmonella typhimurium* *cyd* operon and, for comparison, the *E. coli* sequence.

EXAMPLE 1: MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

S. typhimurium strain F98 is a prototrophic isolate from diseased chickens whose virulence and colonisation characteristics in chickens and mice have been well characterised(5, 6, 9). Spontaneous mutants of this and other strains resistant to nalidixic acid (Nal^r) or spectinomycin (Spc^r) were produced as described previously (39). Insertion mutant AB145 of F98 (11) was produced previously by Tn*phoA* mutagenesis using, as the donor plasmid, pRT733 in *E. coli* SM10. *S. typhimurium* C5 is prototrophic and virulent for mice (20, 28). *S. gallinarum* strain 9 is highly virulent for chickens of all ages (5, 8, 38). *E. coli* K12 strains SY327 lambda *pir*, a lysogen of SY327 ((*lac pro*) *arg E*(Am) *rif nalA recA56*) containing the *pir* gene of plasmid R6K, was the host for transformation of suicide plasmid pGP704 containing the R6K replicon (29) and SM10 *thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km lambda *pir* (37) was used for conjugal transfer of this plasmid. Plasmid vector pBluescript (KS(-) was used for cloning the target gene of the Tn*phoA* mutagenesis. Bacteriophage p22 Ht105/1*int* (35) was used for transduction of markers as described previously (7). Unless indicated otherwise bacterial cultures were made in 10 ml volumes of LB broth (Difco) incubating for 24 h in an orbital incubator (150 revs/min).

DNA manipulations, sequencing and reagents

Chromosomal DNA was prepared as described by Pitcher *et al* (32). Plasmid DNA was prepared using alkaline lysis (34). Restriction endonucleases, T4 DNA ligase and *Taq* DNA polymerase were obtained from Boehringer Mannheim (Germany) and used according to the manufacturer's instructions. DNA fragments cloned in pBluescript KS(-) and DNA from PCR products were sequenced using an oligonucleotide derived from the sequence of the alkaline phosphatase gene in Tn*phoA*. Sequencing was carried out using a cycle sequencing programme with an ABI 373A sequencing system according to the manufacturer's protocols (Applied Biosystems, Foster City, California) and the data analysed using the GCG software package (17).

NADH dehydrogenase assay

The method was essentially that of Archer *et al* (3) with a number of differences. Cells were grown to late log phase (OD = 0.7) in LB broth and were disrupted at -70°C with an X-press (Biox Ltd, Sweden). After removal of cell debris at 10,000 g for 10 min the protein concentrations of the preparations were measured spectrophotometrically (Pierce) and equalised before use.

Growth inhibition assay

The procedure has been described previously (11). Basically, a 24 h LB culture of one strain, resistant to one antibiotic, was inoculated with small numbers of a second strain, resistant to another antibiotic, followed by further incubation with enumeration of the second strain. The initial count of the second strain was ca. 10^3 cfu/ml. Mutants were tested both as the

first and the second strain. Berchieri and Barrow (11) showed that inhibition was not related to resistance to either spectinomycin or nalidixic acid, the antibiotic resistances used in the assay.

Virulence assays

These were essentially following the protocols described by Barrow *et al* (5). Newly hatched chickens were inoculated orally with 0.1 ml volumes and three-week-old birds with 0.3 ml of undiluted cultures or intramuscularly with 0.1 ml of decimal dilutions of cultures. Mice were inoculated orally with 0.05 ml volumes of cultures diluted in LB, while under light anaesthesia, or intravenously with 0.1 ml volumes of cultures diluted similarly. Animals which died or were killed after exceeding humane end points over periods of three weeks were scored. LD_{50} values were estimated (33).

The intestinal invasiveness of *S. gallinarum* 9 Nal^r and its *nuoG* mutant (see results section) was assessed in two groups of chickens by assessing the rate at which organisms accumulated in the liver and spleen in the first three days after oral inoculation. This has recently been found to be a reliable indicator of this characteristic (5). The behaviour of these two strains in the reticuloendothelial system of chickens was assessed by counting inoculated bacteria in the liver, spleen and blood following intravenous inoculation with 10^4 cfu (*S. gallinarum* 9) or 10^6 cfu (*nuoG* mutant). Bacteria were counted on Brilliant Green agar (CM263, Oxoid, Basingstoke, United Kingdom) containing sodium nalidixate (20 μg/ml) and novobiocin (1 μg/ml).

RESULTS

Characterisation of the Tn*phoA* insertion site in *S. typhimurium* F98 AB145

The non-inhibitory (*in vitro*) mutant of *S. typhimurium* F98, namely AB145, was partially rough as indicated by lipopolysaccharide profiles (results not presented). However, sufficient LPS was produced to enable the transposon-associated antibiotic-resistance marker to be transduced to the parent strain using bacteriophage P22. All kanamycin resistant transductants tested showed a similar non-inhibitory phenotype to AB145 (see below). The Tn*phoA* insertion in AB145 was therefore likely to be the mutation responsible for the inability of a stationary phase LB culture to inhibit the growth of *S. typhimurium* F98 Spc^r inoculated into the AB145 culture.

Previously reported work (11) indicated that the Tn*phoA* inactivated gene(s) in AB145 was contained within a 11 kbp *Eco*RV fragment. Initial attempts at cloning the whole fragment into pBluescript (KS(-) were unsuccessful. *Hin*dIII digestion of AB145 chromosome DNA revealed hybridisation with a ca. 3.3. kbp fragment which contained the target gene-TN*phoA* junction. A section of an identical gel, corresponding to the position of this fragment, was excised. The DNA was purified and cloned into the compatible site of plasmid vector pBKS(-) and this was transformed into host strain XL 1-blue. Colonies containing the expected cloned 3.3 kbp *Hin*dIII fragment were identified by digestion of plasmid DNA followed by hybridisation using a ECL-labelled (ECL, Amersham) 1.3 kbp *Eco*RI-*Xho*I DNA fragment derived from the alkaline phosphatase gene of Tn*phoA*.

The nucleotide sequence of the chromosomal fragment adjacent to the upstream Tn*phoA* IS_{50L} was determined by cycle sequencing using a primer as described in the Materials and Methods section to allow sequencing outwards from the negative strand of Tn*phoA*. The result revealed that Tn*phoA* had inserted at nucleotide 1468 of *nuoG*, one of the genes in the *nuo* operon encoding NADH dehydrogenase I. The open reading frame of *nuoG* in *S. typhimurium* has not previously been completely sequenced. It was apparent that the orientation of Tn*phoA* was such that the 5′ end of the alkaline phosphatase gene was upstream from the 3′ end of *nuoG* in AB145. In this orientation transcription of the *phoA* sequence would not have occurred from the *nuo* promoter. The Tn*phoA* mutation in AB145 was therefore transduced by P22 to a *phoN* mutant of F98, which produces white colonies on LB agar containing 40 μg/ml X-P. All kanamycin resistant transductants were also white, indicating no detectable expression of *phoA* from the *nuo* or any other promoter.

Comparison between the *nuoG* and *nuoH* sequences of *S. typhimurium* F98 and *E. coli*

Both strands of a fragment containing parts of *nuoG* gene and a fragment containing *nuoH* gene, that was detected immediately downstream of *nuoG*, were determined by direct PCR sequencing. For this oligonucleotide primers, based on the sequence of *nuoG*, *nuoH* and *nuoI* of *E. coli*, were used to amplify the genes from a colony of *S. typhimurium* F98. The deduced amino acid sequence encoding part of *nuoG*, and the whole of *nuoH*, together with the sequence of the same genes from *E. coli* is shown in Figure 1. The sequence data will appear in the EMBL/GENEBANK Nucleotide Sequence Data Libraries under the accession number L42521.

Comparison of the two gene sequences reveals a high degree of homology, many of the amino acid differences being conservative substitutions. The only major difference between the *E. coli* K-12 and *S. typhimurium* F98 sequences occurs at the 3'-end of the *nuoG* gene. The predicted *Salmonella* protein is 20 amino acids longer than that of *E. coli*. The comparable sequence in *E. coli* K-12 contains non-coding triplets which would result in premature termination of translation of the gene. Comparison of the *nuoH* gene between *E. coli* K-12 and *S. typhimurium* F98 revealed very similar sequences.

NADH and dNADH assays

The results of assessing membrane vesicle preparations of the parent F98 and the *nuoG*::Tn*phoA* for NADH and dNADH oxidase activity are shown in Figure 4. The reduced activity of the *nuoG*::Tn*phoA* mutant against NADH was not great, the residual activity probably being due largely to the activity of NADH dhII. NADH dhII is unable to oxidise dNADH as shown in Figure 4, indicating the NADH dhI activity had been virtually eliminated from the *nuoG* mutant.

Construction of a defined mutation in *nuoG*

A defined mutant of *nuoG* harbouring an insertion of a DNA cassette encoding kanamycin-resistance was constructed (Figure 2). A 1.259 kbp *Eco*RI-*Xba*I fragment of the *nuoG* gene was cloned into the compatible site of suicide vector pGP704. A kanamycin gene cassette, carried by pBSK, was removed with *Eco*RV and *Spe*I. After end-filling, the resulting blunt-ended fragment was inserted into the *Eco*RV site within *nuoG*. The constructed plasmid, pGP704, containing *nuoG* with the kanamycin-resistance cassette insertion, was electroporated into *E. coli* SY327 lambda

pir. Plasmid DNA was prepared and transformed into *E. coli* SM10 lambda *pir* (34) enabling the plasmid carrying the mutated *nuoG* to conjugate back into the wild-type *S. typhimurium* F98 Nal^r. The defined *nuoG* mutant was selected for by allele exchange resulting in a kanamycin resistant, ampicillin-sensitive transcripient. The kanamycin cassette insertion in the *nuoG* gene was confirmed using PCR (data not shown).

Inhibitory activity of AB145 and defined mutants

Mutant AB145 was compared with the parent F98 for the ability of a 24 h LB broth culture to inhibit growth of F98 Spc^r. We also studied the growth inhibition of AB145 by 24 h broth cultures of F98. The results of the former are summarised in Figure 3. The growth curves of the parent and mutant are similar. However, unlike the parent strain (Figure 3b), AB145 failed to inhibit the multiplication of F98 Spc^r inoculated into the culture (Figure 3a). The parent strain was able to prevent multiplication of AB145 when this was added (results not shown). The defined *nuoG* mutant also showed an identical phenotype to the Tn*phoA* mutant, failing to inhibit the growth of strain F98 Spc^r when F98 Spc^r was inoculated into a stationary phase culture of the *nuoG* mutant.

The precise *nuoG* mutation was transferred by P22 transduction into *S. typhimurium* C5 and *S gallinarum* 9. These mutants showed the same non-inhibitory phenotype *in vitro* against Spc^r mutants of the parent strains (results not presented).

Virulence of AB145 and defined *nuoG* mutants for chickens and mice

AB145 was partially rough and not surprisingly was avirulent when inoculated orally into newly-hatched chickens, in contrast to the parent

strain *S. typhimurium* F98 which killed 14/25 birds. To assess the role of *nuo* in virulence, the defined *nuoG*::Km mutation was transduced, using bacteriophage P22, into the parent *S. typhimurium* F98 strain. Analysis of the LPS of the parent F98 strain and the *nuoG*::Km mutant confirmed that these strains were smooth (results not shown). The smooth *nuoG*::Km mutant of *S. typhimurium* F98 was considerably less virulent in chickens than the smooth parent strain (Table 1).

Table 1: Virulence of *Salmonella* strains and *nuoG* mutants for chickens

	Virulence in			
	Newly-hatched chicks		3-week-old chickens	
Serotype	*S. typhimurium*		*S. gallinarum*	
Strain	C5	F98	9	
Route	oral	oral	oral	i/m
Parent strain	26/26[a]	24/26[a]	18/24[a]	<0.38[b]
nuoG mutant	13/27	8/26	0/24	>7.08

[a] Number of chicks died/number inoculated with 10^8 cfu in 0.1 ml

[b] Log_{10} LD_{10} value by intramuscular (i/m) routes

The *nuoG*::km mutation was transduced into *S. gallinarum* strain 9. In comparison to the parental *S. gallinarum* strain 9 the isogenic *nuoG* mutant was highly attenuated for chickens by both oral and parenteral routes of inoculation. There appeared to be little difference in invasiveness to the liver and spleen from the alimentary tract following oral inoculation of chickens with *S. gallinarum* 9 or its *nuoG* derivative. Both strains were found in similar numbers in the caeca soon after infection and appeared in the liver and spleen at similar intervals after inoculation (Table 2).

Table 2. Intestinal invasiveness of *S. gallinarum* 9 and its *nuoG* mutant

Days after infection	Log_{10} viable count/gm of parent strain or mutant in the following organs[a]									
	nuoG mutant					parent				
	Liver	Spleen	Caeca			Liver	Spleen	Caeca		
			contents	mucosa	tonsil			contents	mucosa	tonsil
1	N[b]	N	2.4	2.6	0.8	N	N	3.0	2.2	0.8
2	N	N	N	N	1.4	N	N	0.7	N	2.9
3	0.8	0.7	N	1.9	1.3	1.7	1.6	1.4	3.3	1.8
4	0.9	1.0	N	1.5	1.5	2.0	1.9	1.2	2.4	2.0

[a] Mean of values from three animals

[b] N = $\log_{10} < 0.5$

The higher numbers of the parent strain in these two organs at four days after infection indicated multiplication of this strain whereas none seemed to have occurred of the *nuoG* mutant. This was also observed after intravenous inoculation.

Table 3. Behaviour of *S. gallinarum* 9 and its *nuoG* mutant in the tissues after intravenous inoculation

Days after infection	Log_{10} viable count/gm of parent strain or mutant in the following samples[a]					
	nuoG mutant			parent		
	Liver	Spleen	Blood	Liver	Spleen	Blood
0	4.5	6.3	3.6	2.5	4.3	0.7
2	5.1	5.7	0.8	4.6	5.0	N
4	4.2[c]	5.6	N[b]	4.9[c]	5.4[c]	1.0
7	3.4[c]	5.3[c]	N	5.5[c]	5.0[c]	1.7
10	2.7[c]	4.6[c]	N	5.7[c]	5.6[c]	2.4
14	3.3[c]	4.5[c]	N		Dead	
21	3.3[c]	5.0[c]	N			
35	2.7	4.5	N			
42	1.7	2.6	N			

[a] Mean of values from three animals

[b] N = $\log_{10} < 0.5$

[c] necrotic lesions present in organs

The parent strain multiplied in the liver and spleen until a bacteraemia occurred and the animals died. Despite inoculation of 100 times more organisms of the *nuoG* mutant the chickens remained healthy. This strain persisted in the liver and spleen in considerable numbers during the course of the experiment.

The *nuoG*::Km mutation was also transduced into the mouse-virulent *S. typhimurium* strain C5 and groups of BALB/c mice were orally challenged with doses of 10^6 or 10^8 parental or *nuoG*::Km mutant bacteria. Four out of five mice challenged with 10^6 and twelve out of twelve mice challenged with 10^8 wild-type *S. typhimurium* C5 died. However, all ten mice challenged with 10^6 and seventeen of twenty mice challenged with 10^8 *S. typhimurium* C5 *nuoG*::Km survived the challenge. Mice surviving the *S. typhimurium* C5 challenge harboured bacteria in their livers and spleens and some had small abscesses in these organisms. The number of bacteria per organ showed considerable variation between individual mice and the persistence pattern resembled that seen previously following infection with *purE* mutants (30).

DISCUSSION

This study describes some of the biological characteristics of *nuoG* mutants of *S. typhimurium* and *S. gallinarum* which have defective NADH dehydrogenase I activity. We have demonstrated that such a defect attenuates virulence of these serotypes for mice and chickens and that it abolishes the genus-specific inhibition of growth seen in early stationary phase broth cultures. The mutation was detected while screening Tn*phoA* mutants for their inability to inhibit the multiplication of *S. typhimurium* F98 Nal^r Spc^r when incubated as 24 h broth cultures. The original mutant, AB145, was partially rough. This rough phenotype was likely to

have been selected during conjugation, when the plasmid pRT733 containing Tn*phoA* was introduced. However, it was sufficiently susceptible to bacteriophage P22 to allow retransduction to the parent strain. The phenotype was transferred to all recipients tested, indicating that the transposon insertion, rather than the partially rough phenotype, was responsible for the characteristics of this mutant. Production of a defined mutation showed that the lesion responsible for the inhibitory phenotype was in *nuoG*, situated in the middle of this operon containing fourteen genes (*nuoA-N*). The large number of termination codons between *nuoG* and *nuoH* suggest that translation downstream of *nuoG* may be reduced normally. Whether this contributes to some form of regulation of *nuoH* to *nuoH* is not known. It is unclear why there should be a difference in the length of *nuoG* in *S. typhimurium* F98 and *E. coli*. This may be explained simply by a comparison of a wild-type and laboratory strain. Stop codons could have accumulated in the *E. coli* gene over many years of *in vitro* culture.

Mutants of *S. typhimurium* strains F98 and C5 and of *S. gallinarum* 9 which were *nuoG* showed reduced virulence for chickens and mice. Moreover, introduction of the *nuoG* mutation into *S. gallinarum* produced a great reduction in virulence both by oral and parenteral virulence. In this case the major affected stage of pathogenesis appeared to be the ability to multiply in the reticuloendothelial system rather than intestinal colonization and invasion. The difference in the degree of attenuation between these two serotypes may reflect more fundamental differences in their virulence attributes. Elimination of the virulence plasmid from *S. gallinarum* also attenuates this serotype to a much greater extent than occurs following the same manipulation of *S. typhimurium* (4, 8).

Although mutant AB145 did not produce inhibition of growth in stationary

phase LB broth cultures it was nevertheless inhibitory *in vivo* (11) demonstrating that *in vivo* and *in vitro* inhibition mechanisms are different or are stimulated by different environmental conditions.

Growth suppression in the absence of nutrient starvation could be mediated by inter-bacterial signalling at high bacterial density. Mutations in *nuo* could conceivably affect this in a number of ways. For example, the reduction in aerobic metabolism which would be characteristic of AB145 would result in a higher than normal oxygen concentration present in early stationary phase. Regulatory proteins sensitive to such changes and indicator molecules which reflect such metabolic changes, such as acetyl phosphate (27) or internal cellular pH, could all separately or together be involved in such a mechanism.

The central role of the electron-transport chain in changes that occur in early stationary phase is supported by the fact that a second non-inhibitory mutant of *S. typhimurium* F98 has been found to have an insertion in the *cyd* operon, encoding cytochrome d oxidase. Table 4 shows that this mutant is non-inhibitory as a 24 h broth culture for small numbers of *Stm* F98 Nal^r, even though it appears to be inhibitory *in vivo*.

Table 4. Multiplication over 4 days of *S. typhimurium* F98 Spc^r (Spc^r mutant of parent strain) in 24 h broth culture of *cyd* mutant of *S. typhimurium* F98 Nal^r.

Time (days) after inoc. of challenge strain	Log_{10} viable numbers of *cyd* mutant	Log_{10} viable numbers of challenge strain
0	9.60	3.59
1	9.69	7.53
4	9.78	9.30

EXAMPLE 2: CONSTRUCTION OF A DEFINED MUTATION IN *cydA*

This may be done in two ways:

1. Method 1 is to clone into a suicide plasmid such as pGP704 vector the *cyd* operon (*cydA* and *B*), amplified by the PCR with oligonucleotides 1 and 4. This fragment is then digested with *EcoRV* at base 1242 (in em_ba:eccyd). A kanamycin gene cassette, carried by pBSK, was amplified with oligonucleotides 5 and 6 which have *Kpn*I sites included at their 5′ ends. After end-filling, the resulting blunt-ended fragment was inserted into the *EcoRV* site within *cydA*. This constructed plasmid containing cloned *cydA* and *B* with the kanamycin cassette insertion was electroporated into *E. coli* SY327 λ *pir* (Ref 29). Plasmid DNA was prepared and transformed into *E. coli* SM10 λ *pir* (Ref 37) enabling the plasmid carrying the mutated *cyd* operon to conjugate back into the wild-type *S. typhimurium* strain. The defined mutation was selected for by allele exchange resulting in a kanamycin-resistant, ampicillin-

sensitive transcipient. The insertion is confirmed by PCR using oligonucleotides 1 and 4.

Oligonucleotides from any parts of the sequence may be used to check by PCR whether the gene has been disrupted, for example by insertion of an antibiotic resistance cassette. Oligonucleotides prepared from the extreme ends of the sequence will give a fragment approximately 2750-2800 in size depending on the size of the oligonucleotide. Insertion of a cassette will either disrupt this or will create an enlarged fragment.

2. Method 2 is to amplify single fragments by PCR from the N-terminal end of *cydA* (oligonucleotides 1 and 2) and from the C-terminal end of *cydB* (oligonucleotides 3 and 4). The two fragments have *Kpn*I sites with which they may ligate to each other and *Eco*RI and *Xba*I sites for ligation into pGP704. This plasmid is transferred sequentially into *E. coli* SY327 λ *pir* and *E. coli* SM10 λ *pir*. Allele exchange is used selecting for ampicillin sensitive transcipients. The deletion incorporating part of *cydA* and *cydB* is confirmed by PCR using oligonucleotides 1 and 4.

Oligonucleotide primers for *cydA* and *B* taken from em_ba:eccyd

Primer 1	base 10-29 with *Eco*RI site added to 5′ end
Primer 2	base 1146-1155 with *Kpn*I site added to 5′ end
Primer 3	base 1877-1896 with *Kpn*I site added to 5′ end
Primer 4	base 3582-3601 with *Xba*I site added to 5′ end

Kanamycin cassette oligonucleotides

Primer 5 GAATTCGGTACCCGCTGAGGTCTGCCTCGTGAAGG

Primer 6 GAATTCGGTACCAAAGCCACGTTGTGTCTAAAATC

EXAMPLE 3: CONSTRUCTION OF A DEFINED MUTATION IN *uncH* (ATP SYNTHASE)

Method 2 was followed in an identical way. The oligonucleotides were taken from the *E. coli* sequence deposited with the EMBL nucleotide data library (no em_ba:ecunco1).

N-terminal end {primer 1 base no 1990-2014 *Xba*I site added to 5′ end
{primer 2 base no 2760-2785 *Kpn*I site added to 5′ end
C-terminal end {primer 3 base no 3411-3436 *Kpn*I site added to 5′ end
{primer 4 base no 4045-4069 *Eco*RI site added to 5′ end

ATP synthase is concerned with ATP generation rather than the release of protons back with the cells. The mutant created here is non-inhibitory *in vitro*. In other words, when inoculated in small numbers into a 24 hour broth culture of the parent strain, it does not inhibit growth of the parent strain.

After incubation of the mixture the counts of the parent strain at various times of sampling were as follows:

0d	3×10^2	cfu/ml
1d	2.1×10^4	cfu/ml
2d	6×10^5	cfu/ml
3d	2.7×10^6	cfu/ml

4d	2.5×10^7	cfu/ml
7d	1.3×10^8	cfu/ml

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CLAIMS

1. A vaccine composition comprising an avirulent mutant of a cellular pathogen which colonizes a vertebrate mucosal surface (preferably the gut), the mutant being characterised by having a functional deletion of a gene encoding a protein involved in the electron transport chain or ATP synthase.

2. A composition according to Claim 1 wherein the pathogen is a bacterium.

3. A composition according to Claim 2 wherein the bacterium is an *Escherichia*, *Salmonella* or *Campylobacter* species.

4. A composition according to Claim 3 wherein the mutant is not *Salmonella typhimurium* AB145.

5. A composition according to any one of the preceding claims wherein the pathogen is a pathogen of birds (for example chickens) or bovines (for example calves).

6. A composition according to any one of the preceding claims wherein the said gene is in the *nuo* operon, encoding the multi-subunit enzyme NADH dehydrogenase I, or in the *cyd* operon, encoding cytochrome oxidase.

7. A composition according to Claim 6 wherein the *nuo* gene is *nuoG* or *nuoH*.

8. A composition according to any one of the preceding claims

wherein the mutant inhibits colonization of the gut by pathogens of the same genus but does not inhibit growth of pathogens of the same genus in *in vitro* culture.

9. A method of preventing or ameliorating a disease caused by a cellular pathogen in a vertebrate, the method comprising administering to the vertebrate a vaccine composition according to any one of the preceding claims.

10. A method according to Claim 9 wherein the administration comprises delivering the vaccine composition to the gastrointestinal tract directly.

11. A method according to Claim 10 wherein the vertebrate is a chick and the vaccine composition is sprayed onto its fur.

12. A method according to any one of Claim 9 to 11 wherein the vertebrate is no more than one day old.

13. The *Salmonella typhimurium nuoG* gene or a variant thereof other than as part of the *S. typhimurium* genome.

14. The *Salmonella typhimurium nuoH* gene or a variant thereof other than as part of the *S. typhimurium* genome.

15. A polynucleotide which can be integrated into the *Salmonella typhimurium* genome to cause the functional deletion of the *nuoG* or *nuoH* genes.

16. A *Salmonella* strain having a functional deletion of the *nuoG* or

nuoH genes.

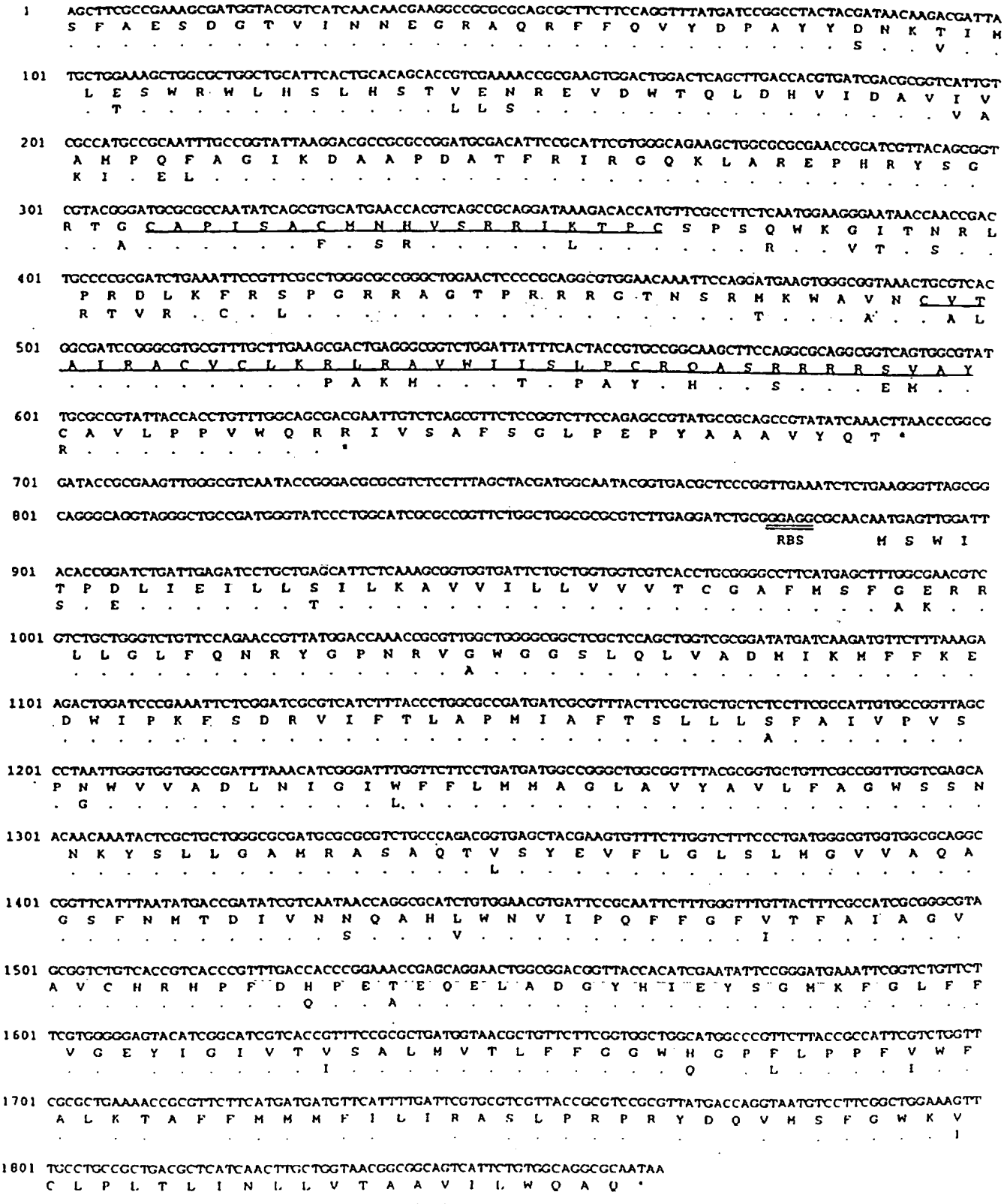


Figure 1

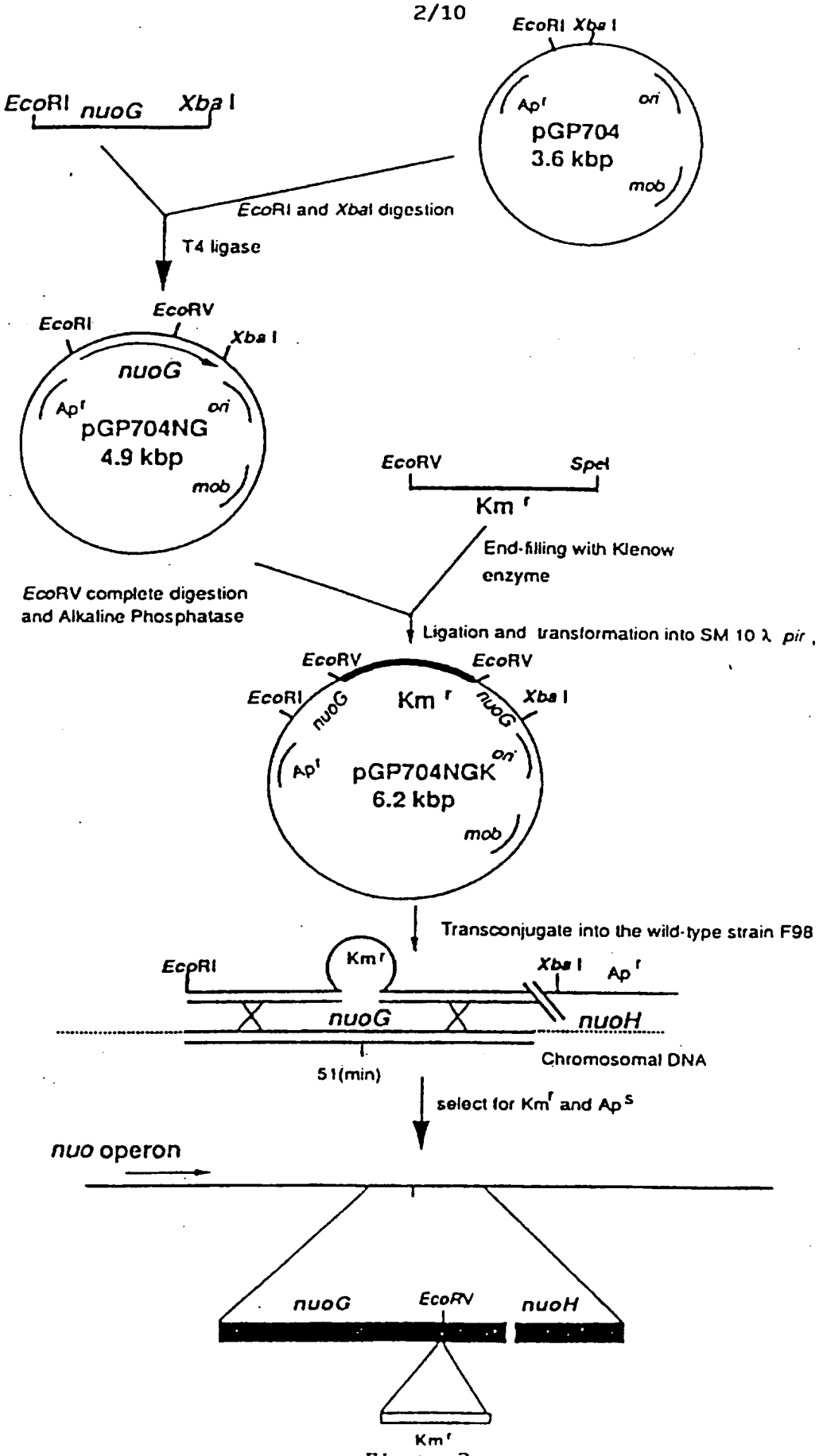


Figure 2

SUBSTITUTE SHEET (RULE 26)

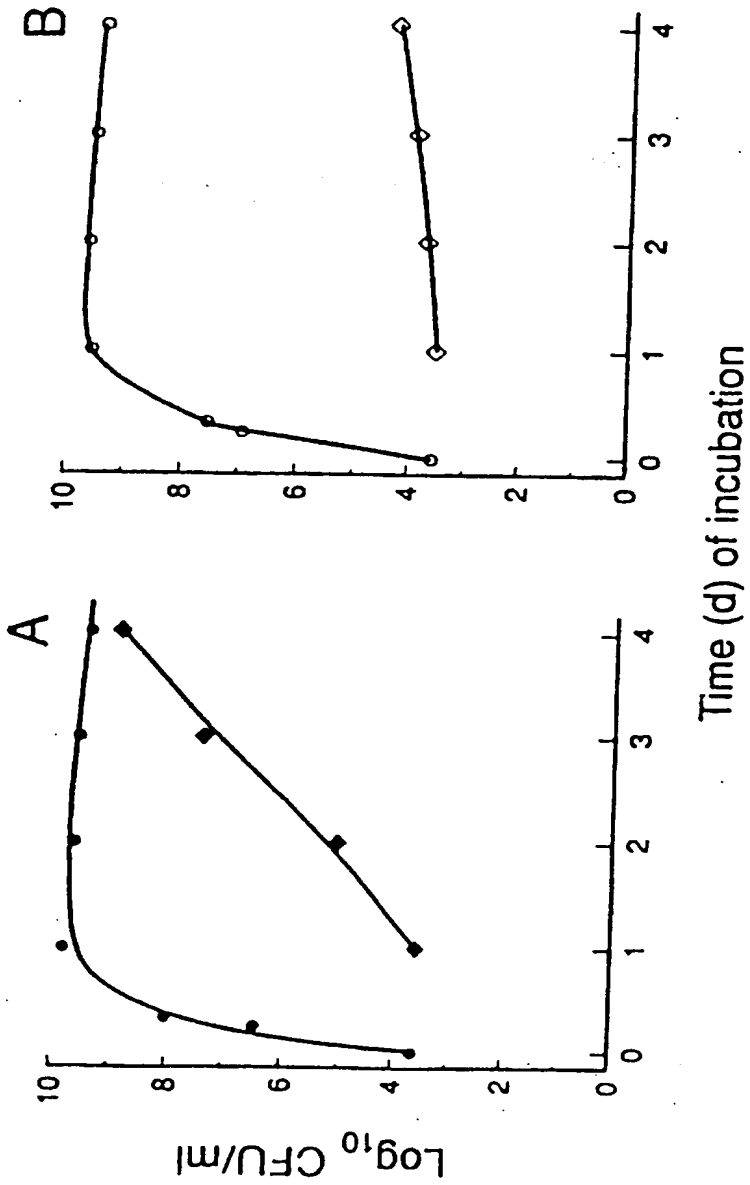
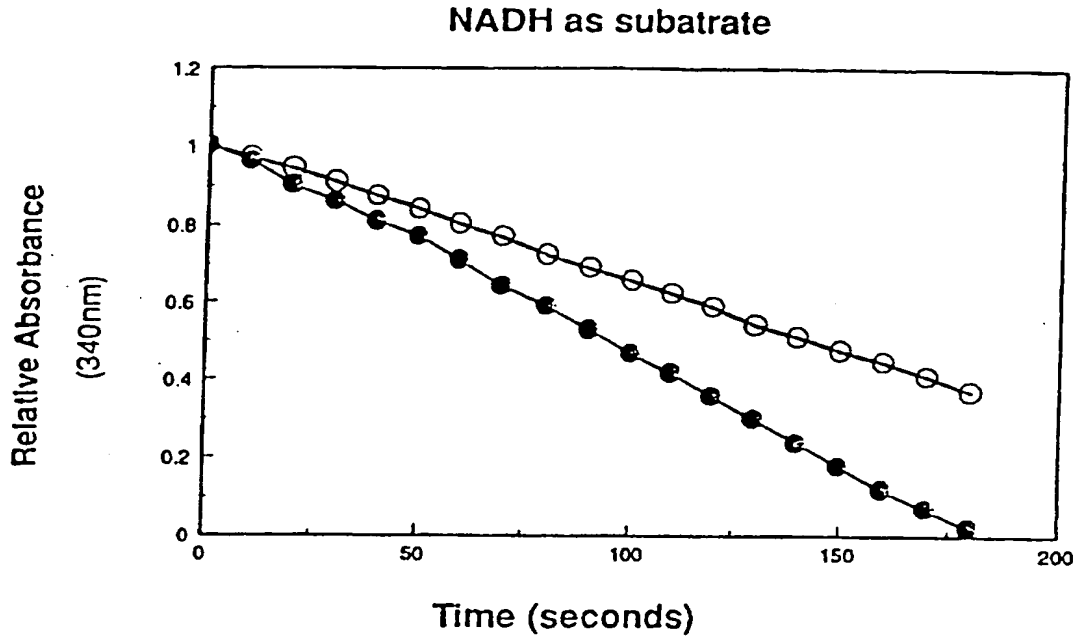


Figure 3



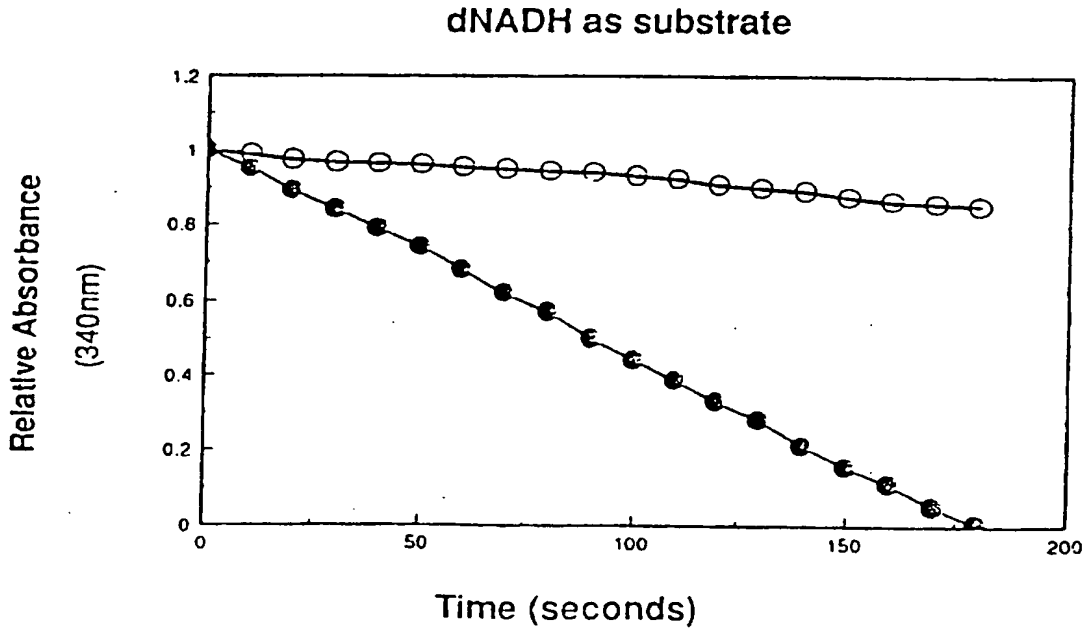


Figure 4

(Nucleotide) FASTA of: gmcyd.seq from: 1 to: 2818 March 29, 1996 13:55

TO: genembl:* Sequences: 737,621 Symbols: 503,641,042 Word Size: 6
Scoring matrix: GenRunData:fastadna.cmp
Constant pamfactor used
Gap creation penalty: 12.0 Gap extension penalty: 4.0

Histogram Key:
Each histogram symbol represents 6627 search set sequences
Each inset symbol represents 1 search set sequences

cydAb Ec of Stm
Stm = gmcyd
E. coli = eccyd

Score	Initl (-)	Initn (+)	
< 4	514	514:	=
8	6	6:	=
12	18	18:	=
16	67	67:	=
20	205	205:	=
24	6983	6983:	==
28	29481	29481:	=====
32	148082	148082:	==========
36	397576	397576:	==========
40	344583	344583:	==========
44	215041	215041:	==========
48	160426	160426:	==========
52	89759	89759:	==========
56	47112	47112:	========
60	20331	20331:	====
64	8340	8340:	==
68	3389	3389:	=
72	2306	2306:	=
76	602	602:	=
80	274	274:	=
84	64	64:	=
88	36	36:	=
92	11	11:	=
96	14	14:	=
100	3	3:	=
104	1	1:	=
108	1	1:	=
112	4	4:	=
116	0	0:	
120	1	0:	-
124	0	0:	
128	0	0:	
132	0	0:	
136	0	0:	
140	0	0:	
144	0	0:	
148	1	0:	- : -
152	0	0:	:
156	0	0:	:
160	0	0:	:
164	0	0:	:
168	0	0:	:
172	0	0:	:
176	0	0:	:
180	1	1:	= : =
184	0	0:	:

Figure 5 (1 of 6)

```
 188      0      0:          :
 192      0      0:          :
 196      1      1:=         :=
 200      0      0:          :
>200      9     11:=         :==========++

Mean score calculations exclude scores greater than 155
 mean initn score:  40.5 (s.d. 6.85)
 mean init1 score:  40.5 (s.d. 6.85)
 1881 scores better than 70 saved, joining threshold: 80

The best scores are:                                          init1 initn  opt..

em_ba:eccyd  J03939 E.coli cytochrome d oxidase subunits ...5057  5186  8529
em_ba:avcydab  M77787 Azotobacter vinelandii cytochrome d...2737  3718  2841
em_ba:s57066  S57066 cydA=cytochrome d oxidase complex su...2737  3718  2841
em_ba:s63811  S63811 appC=cytochrome d oxidase, subunit I...2124  3327  2202

gmcyd.seq
em_ba:eccyd

ID   ECCYD      standard; DNA; PRO; 3845 BP.
AC   J03939;
DT   04-OCT-1988 (Rel. 17, Created)
DT   15-JUL-1994 (Rel. 40, Last updated, Version 5)
DE   E.coli cytochrome d oxidase subunits I and II (cyd) genes, complete
DE   cds. . . .


SCORES            Init1:  5057 Initn:  5186 Opt:  8529
            86.1% identity in 2814 bp overlap

                                        10        20        30
gmcyd.                              GNTGNTAGANCNNTTCTAAANGGGTTCACTCCTCGGA
                                    ||:|::|||||| :|||||||||| ||||||
eccyd  TCAAACAAATTTCCATTGGGGCATGCGTGTGACCCTTTCTAACGGGGTTCACT-CTCGGA
       890       900       910       920       930       940

        40        50        60        70        80        90
gmcyd. GTCTTCATGCGATGAGCAAGGAGTCATGATGTTAGATATAGTCGAACTGTCGCGCTTACA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eccyd  GTCTTCATGCGATGAGCAAGGAGTCATGATGTTAGATATAGTCGAACTGTCGCGCTTACA
        950       960       970       980       990      1000

       100       110       120       130       140       150
gmcyd. GTTTGCCTTGACCGCGATGTACCACTTCCTGTTTGTGCCGCTAACGCTCGGTATGGCGTT
       |||||||||||||||||||||||||||| |||||||| || ||||||||||||||||||
eccyd  GTTTGCCTTGACCGCGATGTACCACTTCCTTTTTGTGCCACTGACGCTCGGTATGGCGTT
       1010      1020      1030      1040      1050      1060

       160       170       180       190       200       210
gmcyd. CCTGCTGGCCATTATGGAAACGGTATACGTCCTTTCCGGCAAACAGATTTATAAAGATAT
       |||||||||||||||||||||||| |||||||| ||||||||||||||||||||||||||
eccyd  CCTGCTGGCCATTATGGAAACGGTCTACGTCCTCTCCGGCAAACAGATTTATAAAGATAT
       1070      1080      1090      1100      1110      1120

       220       230       240       250       260       270
gmcyd. GACCAAGTTCTGGGGCAAGTTGTTTGGTATCAACTTAGCTCTGGGTGTGGGTACCGGTTT
       |||||||||||||||||||||||||||||||| |||||||||||| ||||||| |
eccyd  GACCAAGTTCTGGGGCAAGTTGTTTGGTATCAACTTCGCTCTGGGTGTGGCTACCGGTCT
       1130      1140      1150      1160      1170      1180

       280       290       300       310       320       330
gmcyd. GACCATGGAGTTCCAGTTCGGGACAAACTGGTCGTACTACTCCCACTATTTGGGGGACAT
       |||||||||||||||||||||||| |||||||| ||||| ||||||||| | ||||| ||
```

Figure 5 (2 of 6)

```
eccyd  GACCATGGAGTTCCAGTTCGGGACTAACTGGTCTTACTATTCCCACTATGTAGGGGATAT
       1190       1200       1210       1220       1230       1240

       340        350        360        370        380        390
gmcyd. CTCCGGTGCTCCGCTGTCCATCGANGGGGTGATGTCCTTCTTCCNCGAAGCCACCTTTGT
       || |||||| |||||| | |||||:|| ||||| |||||||||:|||| ||||||||||
eccyd  CTTCGGTGCGCCGCTGGCAATCGAAGGTCTGATGGCCTTCTTCCTCGAATCCACCTTTGT
       1250       1260       1270       1280       1290       1300

       400        410        420        430        440        450
gmcyd. AGGTCTGTTCTTCTTCGGCTGGGATCGTCTGAGTAAAGTTCAGCATATGTGCGTCACCTG
       ||||||||||||||||||| |||||||||||| |||||||||||||||||||| ||||||||
eccyd  AGGTCTGTTCTTCTTCGGTTGGGATCGTCTGGGTAAAGTTCAGCATATGTGTGTCACCTG
       1310       1320       1330       1340       1350       1360

       460        470        480        490        500        510
gmcyd. GCTGGTGGCTCTGGGGTCCAACCTCTCCGCGTTGTGGATTCTGGTAGCGAACGGCTGGAT
       |||||||||| || || || ||||| ||||| |||||||||||||| ||||||||||||||
eccyd  GCTGGTGGCGCTCGGTTCAAACCTGTCCGCACTGTGGATTCTGGTTGCGAACGGCTGGAT
       1370       1380       1390       1400       1410       1420

       520        530        540        550        560        570
gmcyd. GCAAAACCCAATCGCTCCGGATNTCAATTTCGAAACCATGCGTATGGAAATGGTGAGCTT
       |||||||||||||||| | |||:|||| || ||||| |||||||||| ||||||||||
eccyd  GCAAAACCCAATCGCGTCCGATTTCAACTTTGAAACTATGCGTATGGAGATGGTGAGCTT
       1430       1440       1450       1460       1470       1480

       580        590        600        610        620        630
gmcyd. CTCTGAACTGGTGCTGAACCCGGTAGCACAGGTGAAATTTGTTCACACTGTGGCGTCCGG
       ||| || |||||||| |||||||| || |||||||||| |||||||||| ||||| ||
eccyd  CTCCGAGCTGGTGCTTAACCCGGTTGCTCAGGTGAAATTCGTTCACACTGTAGCGTCTGG
       1490       1500       1510       1520       1530       1540

       640        650        660        670        680        690
gmcyd. CTATGTCACCGGCGCGATGTTCATCCTCGGTATCAGCGCTTACTACATGCTGAAAGGTCG
       ||||| || |||||||||||||||||||||||||| | || ||||||||||||||||
eccyd  TTATGTGACTGGCGCGATGTTCATCCTCGGTATCAGCGCATGGTATATGCTGAAAGGTCG
       1550       1560       1570       1580       1690       1600

       700        710        720        730        740        750
gmcyd. TGACTTCGCCTTTGCTAAACGCTCCTTTGCTATTGCCGCCAGCTTCGGTATGGCTGCCGT
       |||||| ||||| |||||||||||||||||||| || ||||||||||||||||||| ||
eccyd  TGACTTGGCCTTCGCTAAACGCTCCTTTGCTATCGCTGCCAGCTTCGGTATGGCTGCTGT
       1610       1620       1630       1640       1650       1660

       760        770        780        790        800        810
gmcyd. ACTGTCCGTTATCGTACTCGGCGACGAATCCGGTTACGAAATGGGCGACGTGCAGAAAAC
       ||||| ||||| || || || || |||||||| ||||||||||||||||||||||||||
eccyd  TCTGTCTGTTATTGTTCTGGGTGATGAATCCGGCTACGAAATGGGCGACGTGCAGAAAAC
       1670       1680       1690       1700       1710       1720

       820        830        840        850        860        870
gmcyd. CAAGCTCGCTGCGATTGAAGCTGAATGGGAAACGCAACCTGCTCCGGCCTCCTTTACCTT
       ||| || ||||| |||||||| || |||||||||||||||| || ||  |||||||  |
eccyd  CAAACTGGCTGCTATTGAAGCCGAGTGGGAAACGCAACCTGCGCCTGCTGCCTTTACTCT
       1730       1740       1750       1760       1770       1780

       880        890        900        910        920        930
gmcyd. GTTCGGTATTCCTGACCAGGACAAACAGGAAAACCATCTGGCGATTCAGATCCCTTATGC
       |||||| |||||||| |||||  |  ||  ||| |  | |||||||||||||||| ||
eccyd  GTTCGGCATTCCTGATCAGGAAGAGGAGACGAACAAATTTGCGATTCAGATCCCTTACGC
       1790       1800       1810       1820       1830       1840

       940        950        960        970        980        990
gmcyd. GCTCGGTATTATCGCTACGCGTTCCGTGGACACGCCGGTTATTGGACTGAAAGATCTCAT
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Figure 5 (3 of 6)

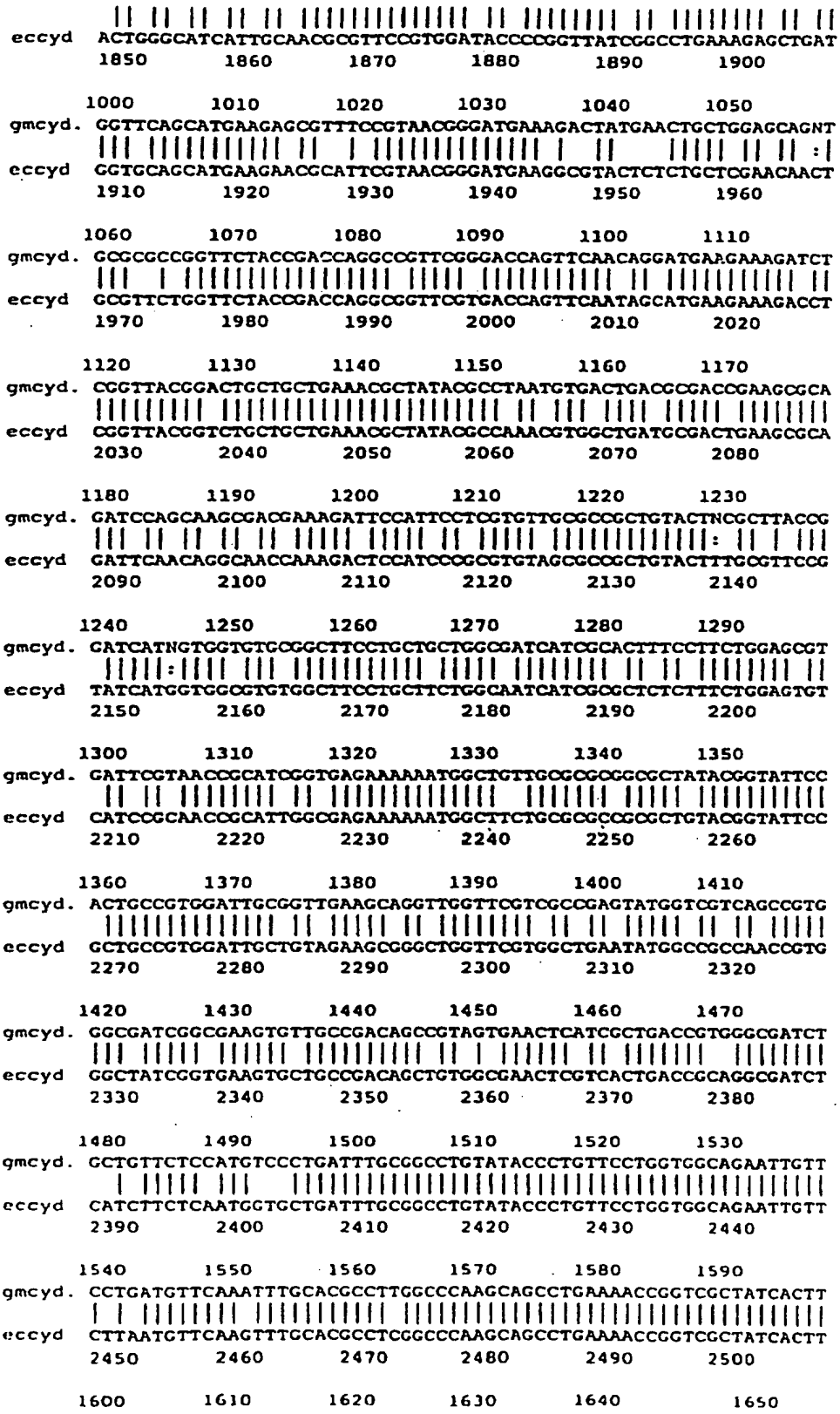


Figure 5 (4 of 6)

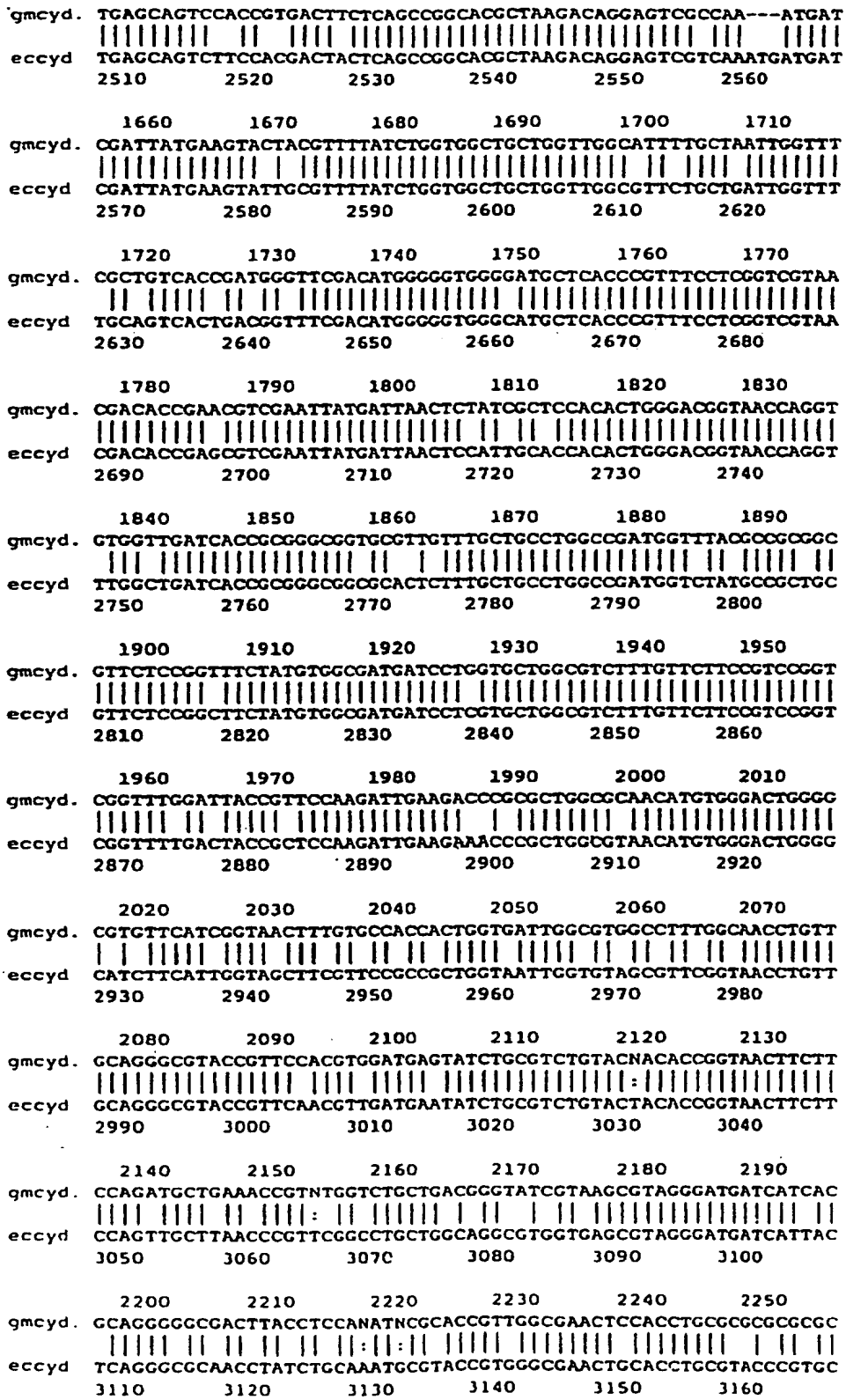
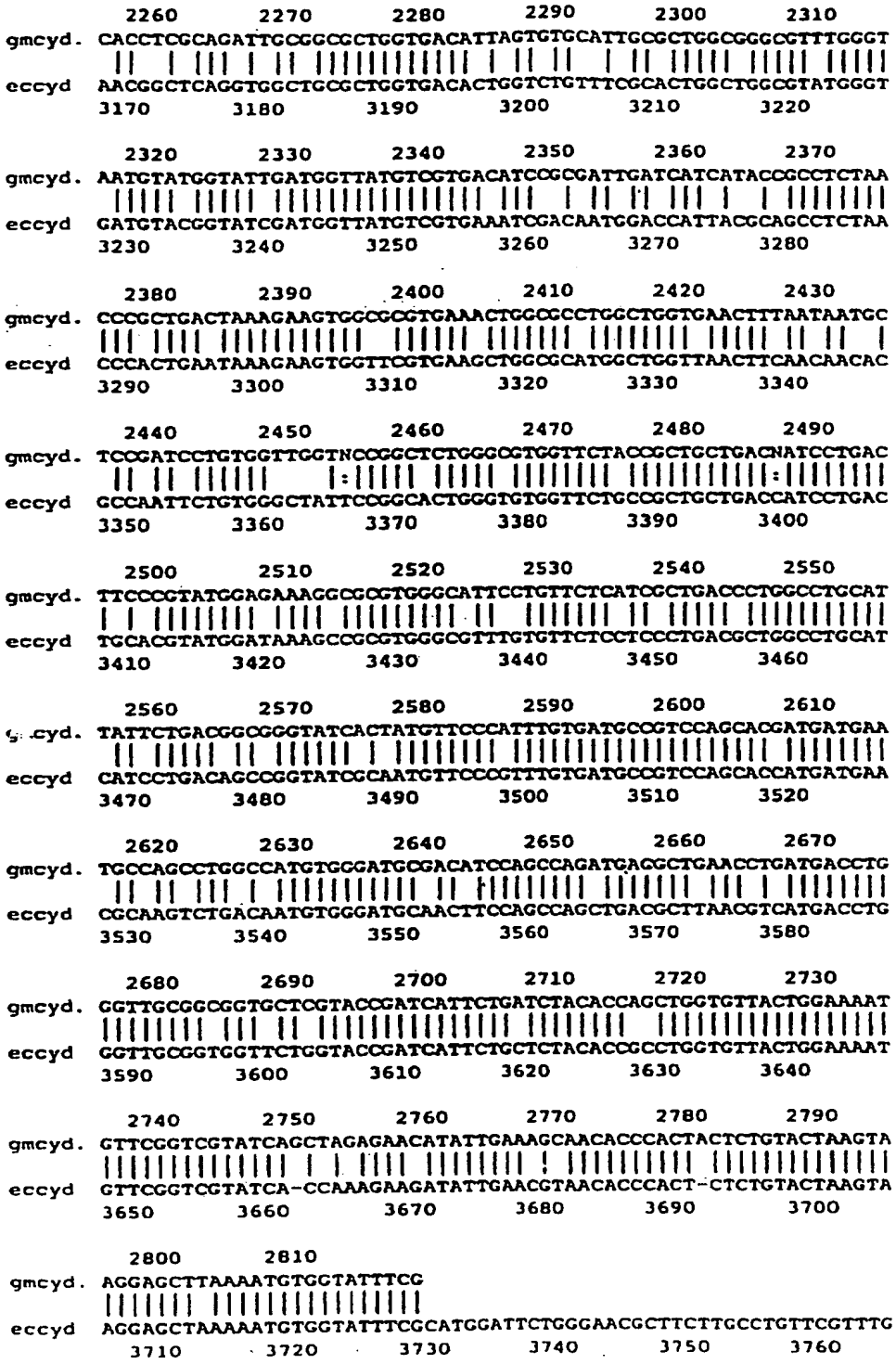


Figure 5 (5 of 6)




```
gmcyd.seq
em_ba:avcydab

ID   AVCYDAB     standard; DNA; PRO; 3387 BP.
```

Figure 5 (6 of 6)

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/106, 39/108, 39/112, C12N 9/02 // (C12N 9/02, C12R 1:42)

A3

(11) International Publication Number: **WO 98/02552**

(43) International Publication Date: 22 January 1998 (22.01.98)

(21) International Application Number: PCT/GB97/01837

(22) International Filing Date: 8 July 1997 (08.07.97)

(30) Priority Data:
9614618.8 11 July 1996 (11.07.96) GB

(71) Applicant *(for all designated States except US)*: INSTITUTE FOR ANIMAL HEALTH LIMITED [GB/GB]; Compton, Newbury, Berkshire RG20 7NN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BARROW, Paul, Andrew [GB/GB]; (GB). TURNER, Arthur, Keith [GB/GB]; Institute for Animal Health Limited, Compton, Newbury, Berkshire RG20 7NN (GB).

(74) Agent: BASSETT, Richard; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
5 March 1998 (05.03.98)

(54) Title: VACCINE COMPOSITIONS

(57) Abstract

A vaccine composition comprising an avirulent mutant of a cellular pathogen which colonizes the vertebrate gut, the mutant being characterised by having a functional deletion of a gene encoding a protein involved in the electron transport chain or ATP synthase. The pathogen may be *Salmonella* or *E. coli*. The vertebrate may be calves or chicks. The gene may be a *nuo* (encoding a sub-unit of NADH dehydrogenase I) or a *cyd* (encoding a cytochrome) gene. The mutants provoke an immune response and also inhibit colonization of the gut by other pathogens.

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INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/GB 97/01837

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/106 A61K39/108 A61K39/112 C12N9/02 //(C12N9/02, C12R1:42)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C. DAWN ARCHER AND THOMAS ELLIOTT: "Transcriptional Control of the nuo Operon Which Encodes the Energy-Conserving NADH Dehydrogenase of Salmonella typhimurium" JOURNAL OF BACTERIOLOGY, vol. 177, no. 9, May 1995, pages 2335-2342, XP002047680	13,14,16
A	see the whole document	1-12

X	A. BERCHIERI JR AND P. A. BARROW: "In vitro characterization of intra-generic inhibition of growth in Salmonella typhimurium" JOURNAL OF GENERAL MICROBIOLOGY, vol. 137, 1991, pages 2147-2153, XP002047681 cited in the application	16
A	see the whole document	4

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[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1997	16.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mennessier, T

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INTERNATIONAL SEARCH REPORT

Inten. ...nal Application No
PCT/GB 97/01837

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 142521 Salmonella typhimurium; nuoG gene; nuoH gene, 18 July 1995 XP002048944 see abstract & BARBER L.Z., FRAENKEL G., DOUGAN G., BARROW P.A:: "The nuo locus in Salmonella typhimurium contributes to the genus-specific inhibit cultures and to virulence" .,	13,14
X,P	DATABASE EMBL Q60010 NADH dehydrogenase subunit, nuoH, 1 November 1996 XP002048960 see abstract & BARBER L.Z., FRAENKEL G., DOUGAN G., BARROW P.A.: .,	13,14
A	G. NEIL GREEN, HONG FANG, RUEY-JEN LIN, GAIL NEWTON, MICHAEL MATHER, CHRISTOS D. GEORGIOU AND ROBERT B. GENNIS: "The Nucleotide Sequence of the cyd Locus Encoding the Two Subunits of the Cytochrome d terminal oxidase Complex of Escherichia coli" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 26, 15 September 1988, pages 13138-13143, XP002047682 see the whole document	1-6,8-12
A	BIRGIT M. PRÜSS, JENNIFER M. NELMS, CHANKYU PARK, AND ALAN J. WOLFE: "Mutations in NADH:Ubiquinone Oxidoreductase of Escherichia coli Affects Growth on Mixed Amino Acids" JOURNAL OF BACTERIOLOGY, vol. 176, no. 8, April 1994, pages 2143-2150, XP002047683 cited in the application see the whole document	1-12

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INTERNATIONAL SEARCH REPORT

Inten ɔnal Application No
PCT/GB 97/01837

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. DAWN ARCHER, XIUHUA WANG, AND THOMAS ELLIOTT: "Mutants defective in the energy-conserving NADH dehydrogenase of Salmonella typhimurium identified by a decrease in energy-dependent proteolysis after carbon starvation" PROC. NATL. ACAD. SCI. USA, vol. 90, November 1993, pages 9877-9881, XP002047684 see the whole document ---	1-14,16
A	THOMAS M. DEVLIN: "Textbook of Biochemistry With Clinical Correlations" 1992 , WILEY-LISS, INC. , NEW-YORK XP002047685 see page 285 - page 286 -----	1-5,8-12

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 97/01837

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-12 and 16
because they relate to subject matter not required to be searched by this Authority, namely:
Vaccine compositions comprising an avirulent mutant of a cellular pathogen having a functional deletion of a gene encoding a protein involved in the electron transport chain or ATP synthase. Methods of treatment using said compositions. Mutant strains to be used in said compositions.

2. [X] Claims Nos.: 13-14
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Salmonella typhimurium nuoG and nuoH genes, or variant thereof.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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(51) International Patent Classification 6:
A61K 39/106, 39/108, 39/112, C12N 9/02
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(11) International Publication Number: WO 98/02552

(43) International Publication Date: 22 January 1998 (22.01.98)

(21) International Application Number: PCT/GB97/01837

(22) International Filing Date: 8 July 1997 (08.07.97)

(30) Priority Data:
9614618.8 11 July 1996 (11.07.96) GB

(71) Applicant *(for all designated States except US)*: INSTITUTE FOR ANIMAL HEALTH LIMITED [GB/GB]; Compton, Newbury, Berkshire RG20 7NN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BARROW, Paul, Andrew [GB/GB]; (GB). TURNER, Arthur, Keith [GB/GB]; Institute for Animal Health Limited, Compton, Newbury, Berkshire RG20 7NN (GB).

(74) Agent: BASSETT, Richard; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
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(88) Date of publication of the international search report:
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(88) Date of publication of the revised version of the international search report:
16 July 1998 (16.07.98)

(54) Title: VACCINE COMPOSITIONS

(57) Abstract

A vaccine composition comprising an avirulent mutant of a cellular pathogen which colonizes the vertebrate gut, the mutant being characterised by having a functional deletion of a gene encoding a protein involved in the electron transport chain or ATP synthase. The pathogen may be *Salmonella* or *E. coli*. The vertebrate may be calves or chicks. The gene may be a *nuo* (encoding a sub-unit of NADH dehydrogenase I) or a *cyd* (encoding a cytochrome) gene. The mutants provoke an immune response and also inhibit colonization of the gut by other pathogens.

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 97/01837

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/106 A61K39/108 A61K39/112 C12N9/02 //(C12N9/02, C12R1:42)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C. DAWN ARCHER AND THOMAS ELLIOTT: "Transcriptional Control of the nuo Operon Which Encodes the Energy-Conserving NADH Dehydrogenase of Salmonella typhimurium" JOURNAL OF BACTERIOLOGY, vol. 177, no. 9, May 1995, pages 2335-2342, XP002047680	13,14,16
A	see the whole document	1-12

X	A. BERCHIERI JR AND P. A. BARROW: "In vitro characterization of intra-generic inhibition of growth in Salmonella typhimurium" JOURNAL OF GENERAL MICROBIOLOGY, vol. 137, 1991, pages 2147-2153, XP002047681 cited in the application	16
A	see the whole document	4

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1997	13. 05. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mennessier, T

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 142521 Salmonella typhimurium; nuoG gene; nuoH gene, 18 July 1995 XP002048944 see abstract & BARBER L.Z., FRAENKEL G., DOUGAN G., BARROW P.A.: "The nuo locus in Salmonella typhimurium contributes to the genus-specific inhibit cultures and to virulence" .,	13,14
X,P	DATABASE EMBL Q60010 NADH dehydrogenase subunit, nuoH, 1 November 1996 XP002048960 see abstract & BARBER L.Z., FRAENKEL G., DOUGAN G., BARROW P.A.: .,	13,14
A	G. NEIL GREEN, HONG FANG, RUEY-JEN LIN, GAIL NEWTON, MICHAEL MATHER, CHRISTOS D. GEORGIOU AND ROBERT B. GENNIS: "The Nucleotide Sequence of the cyd Locus Encoding the Two Subunits of the Cytochrome d terminal oxidase Complex of Escherichia coli" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 26, 15 September 1988, pages 13138-13143, XP002047682 see the whole document	1-6,8-12
A	BIRGIT M. PRÜSS, JENNIFER M. NELMS, CHANKYU PARK, AND ALAN J. WOLFE: "Mutations in NADH:Ubiquinone Oxidoreductase of Escherichia coli Affects Growth on Mixed Amino Acids" JOURNAL OF BACTERIOLOGY, vol. 176, no. 8, April 1994, pages 2143-2150, XP002047683 cited in the application see the whole document	1-12

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 97/01837

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. DAWN ARCHER, XIUHUA WANG, AND THOMAS ELLIOTT: "Mutants defective in the energy-conserving NADH dehydrogenase of Salmonella typhimurium identified by a decrease in energy-dependent proteolysis after carbon starvation" PROC. NATL. ACAD. SCI. USA, vol. 90, November 1993, pages 9877-9881, XP002047684 see the whole document ---	1-14,16
A	THOMAS M. DEVLIN: "Textbook of Biochemistry With Clinical Correlations" 1992 , WILEY-LISS, INC. , NEW-YORK XP002047685 see page 285 - page 286 -----	1-5,8-12

9

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 97/01837

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 15
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
As it cannot be derivated from the funtional definition given in claim 15 considered in the light of the description which structural features should be shared by the claimed polynucleotides, it has not been possible to carry out a meaningful search with respect to said claim.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ GB 97/01837

FURTHER INFORMATION CONTINUED FROM PCT/ISA/

1. Claims: 1-12 and 16
   Vaccine compositions comprising an avirulent mutant of a cellular pathogen having a functional deletion of a gene encoding a protein involved in the electron transport chain or ATP synthase. Methods of treatment using said compositions. Mutant strains to be used in said compositions.

2. Claims: 13-14
   Salmonella typhimurium nuoG and nuoH genes, or variant thereof.